The rejection of claims 59, 60, 64, and 65 under 35 U.S.C. § 102(b) as being anticipated by Takase et al., "Genes Encoding Two Lipoproteins in the *leuS-dacA* Region of the *Escherichia coli* Chromosome," J. Bac. 169:5692-5699 (1987) ("Takase") is respectfully traversed. Takase relates to the coding of two lipoproteins by two genes, *rlpA* and *rlpB*, located in the *leu*S-*dac*A region on the *Escherichia coli* chromosome. The *rlpA* gene encodes for a lipoprotein having molecular weight of 36K. Figure 6 of the reference details the sequence of the 36K lipoprotein gene *rlpA* and its 5'- and 3'- flanking regions and the amino acid sequences deduced from the nucleotide sequence. The position of the PTO is that this sequence matches that of the sequence encoding the claimed δ subunit. Applicant respectfully disagrees. The sequence disclosed in Figure 6 of Takase, a sequence of 1408 base pairs, is not the holA sequence of the present invention. In contrast, it is the *rlpA* gene. Takase also discloses a *rlpB* gene of the *E. coli* chromosome. At the end of the sequence of the *rlpB* gene shown in Figure 7, the last 230 base pairs, which were not discussed, constitute a sequence that encodes the first 20-25% of the holA gene sequence. Takase did not recognize this to be an open reading frame of a putative unknown gene, nor did the reference disclose the complete sequence of the holA gene (see diagram attached as Exhibit A showing the overlap between the disclosed *rlpB* gene of Takase and the holA gene encoding the claimed δ subunit). Thus, the sequences disclosed in Takase are distinct from the holA gene. There is no sequence in Takase relevant to the claimed invention other than the first 20-25% of the holA gene sequence. Accordingly, the δ protein subunit of polymerase III holoenzyme and the gene encoding the δ protein subunit of the polymerase III holoenzyme of the present invention are not disclosed by Takase. Moreover, Takase does not disclose the claimed expression system or host cell.

Further, the burden is on the PTO to establish that the sequences disclosed in Takase anticipate the present invention. As noted above and in view of the comments below, the PTO has failed to meet this burden and, in fact, cannot meet this burden. The two sequences disclosed in Takase (i.e., Figures 6 and 7) are not the complete sequence of the holA gene and, therefore, Takase does not disclose the δ protein subunit of the polymerase III holoenzyme or the gene encoding the δ protein subunit of the polymerase III holoenzyme.

In addition, the MPSearch result attached to the August 27, 1999, office action supports applicant's argument that Takase fails to disclose the nucleotide or protein sequences for the entire δ subunit of polymerase II holoenzyme. In particular, the PTO relies upon the MPSearch result, identified as Accession No. M94267, to show that the nucleotide sequence disclosed in Takase is identical to the claimed nucleotide sequence of the present

application. However, the sequence of the MPSearch result relied on by the PTO (i.e., Accession No. M94267) is not the same sequence as either of those disclosed in Takase. In particular, the sequence of the MPSearch result correlates to Reference 2 of the M94267 entry, i.e., Carter et al., "Molecular Cloning, Sequencing, and Overexpression of the Structural Gene Encoding the Delta Subunit of Escherichia coli DNA Polymerase III Holoenzyme," J. Bacteriology 174:7013-7025 (1992) ("Carter"), which was published on October 29, 1992 (i.e., after the effective filing date (January 24, 1992) of the present application). This can be confirmed by review of the "reference" categories in the MPSearch entry. For example, for Reference 2 (i.e., Carter), the reference category states "REFERENCE 2 (bases 1 to 1147)." The length of the sequence of Accession No. M94267 is 1147 base pairs and, thus, Carter discloses the sequence of Accession No. M94267 (see Figure 5 in the copy of Carter attached hereto as Exhibit B). In contrast, for Reference 1 (i.e., Takase), the reference category states "REFERENCE 1 (sites)," i.e., Takase does not disclose the sequence of Accession No. M94267 and is cited only for certain sites in the sequence. Moreover, the MPSearch result relied on by the PTO indicates that there is a conflict in the sequence listed for Accession No. M94267 and the sequence disclosed in Takase (see "conflict" category of MPSearch Entry).

Further, as illustrated in the attached e-mail from GSDB, the MPSearch result (i.e., Accession No. M94267) relied on by the PTO was first released to the public on November 3, 1992 (Exhibit C), i.e., after the effective filing date (January 24, 1992) of the present application. The "two sequences [of Takase] are distinct from the sequence in accession number M94267" (see Exhibit C). The earliest release date associated with Accession No. M94267 is September 5, 1989, which, as noted in Exhibit C, relates to the Takase reference. As noted above and in Exhibit C, Takase does not disclose the sequence of Accession No. M94267, but discloses two different sequences (*rlpA* and *rlpB*). The sequence in Accession No. M94267 was not published as part of Takase. Moreover, the sequence in Accession No. M94267 was not even deposited into GSDB/GenBank until May 13, 1992, i.e., after the effective filing date of the present application. As a result, the MPSearch result itself (i.e., Accession No. M94267) cannot be prior art with respect to the claimed invention and any rejection based on this MPSearch result should be withdrawn.

The burden is on the PTO to establish that the sequence disclosed in the MPSearch result relied upon is entitled to the same date for prior art purposes as Takase. The PTO has failed to meet this burden and, in fact, cannot meet this burden in view of Exhibit C and the previous remarks. Since Takase does not disclose the entire δ protein subunit nor the

entire sequence encoding the δ protein subunit and the MPSearch result is not prior art, there is no basis for an anticipation rejection and the rejection based on Takase must be withdrawn.

The rejection of claims 54, 55, 57-60, 64, and 65 under 35 U.S.C. § 103(a) for obviousness over Takase is respectfully traversed. As stated above, Takase does not disclose the *entire* specified isolated δ protein subunit of polymerase III holoenzyme, nor the *entire* gene encoding that protein. In particular, Takase discloses only a short portion of the gene encoding the δ protein subunit in Figure 7. In addition, Takase provides no motivation to determine the sequence of the remainder of the gene. Specifically, Takase failed to identify the open reading frame of the gene for the δ protein subunit of polymerase III holoenzyme and, therefore, provides no motivation or suggestion to determine the remainder of the gene encoding the δ protein subunit. Further, the focus of Takase is on two genes, *rlpA* and *rlpB*, which are different from the gene encoding the δ protein subunit of polymerase III holoenzyme. As a result, Takase provides no motivation with respect to determining the sequence of the gene encoding the δ protein subunit of polymerase III holoenzyme.

Moreover, the MPSearch result relied upon by the PTO is not prior art with respect to the claimed invention, as described above. Therefore, the rejection based on Takase is improper and should be withdrawn.

In view of the all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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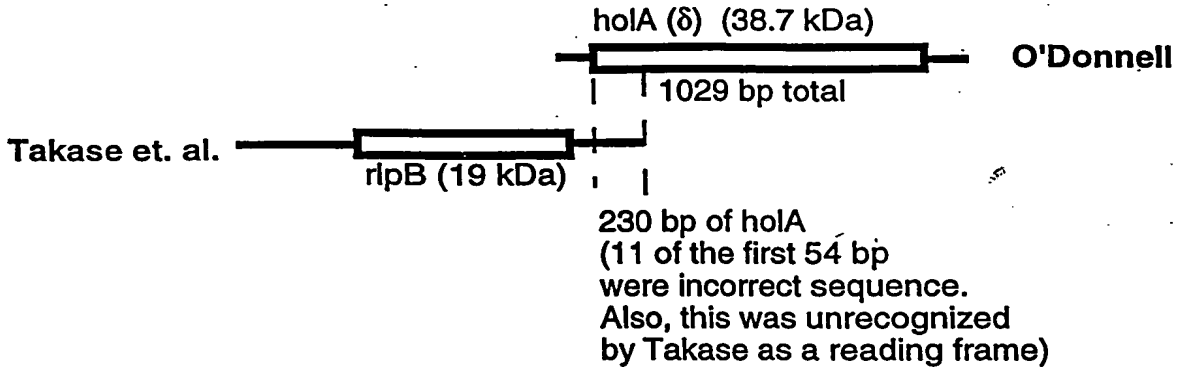
Cheryl S. Mascitti

The holA gene encoding δ



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Molecular Cloning, Sequencing, and Overexpression of the Structural Gene Encoding the δ Subunit of *Escherichia coli* DNA Polymerase III Holoenzyme

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Using an oligonucleotide hybridization probe, we have mapped the structural gene for the δ subunit of *Escherichia coli* DNA polymerase III holoenzyme to 14.6 centisomes of the chromosome. This gene, designated *holA*, was cloned and sequenced. The sequence of *holA* matches precisely four amino acid sequences obtained for the amino terminus of δ and three internal tryptic peptides. A *holA*-overproducing plasmid that directs the expression of δ up to 4% of the soluble protein was constructed. Sequence analysis of *holA* revealed a 1,029-bp open reading frame that encodes a protein with a predicted molecular mass of 38,703 Da. *holA* may reside downstream of *ripB* in an operon, perhaps representing yet another link between structural genes for the DNA polymerase III holoenzyme and proteins involved in membrane biogenesis. These and other features are discussed in terms of genetic regulation of δ-subunit synthesis.

DNA polymerase III (Pol III) holoenzyme (referred to here as holoenzyme) is the major replicative complex of *Escherichia coli*. It contains a core DNA Pol III plus seven auxiliary proteins that confer upon the holoenzyme special properties that distinguish it from simpler polymerases not devoted to chromosomal replication (for reviews, see references 27, 37, and 47). These properties include extreme processivity and a high rate of nucleotide incorporation, resistance to physiological levels of salt, a preference for a single-stranded DNA-binding protein-coated template, and the ability to communicate with components of the replisome to effect coordinated replication of leading and lagging strands of the replication fork (27, 37, 73, 74, 77).

Four different forms of DNA Pol III have been purified. The core Pol III is composed of the catalytic α subunit (*dnaE*), ε (*dnaQ*), the 3′-to-5′ proofreading subunit, and θ. The dimeric Pol III′ is formed upon addition of the τ subunit (*dnaX*) (35, 61) to Pol III. Pol III′ is distinguished from the core by its 10-fold higher processivity (8, 9) and stimulation by physiological concentrations of spermidine (8). Addition of the γδ complex (γ, δ, δ′, χ, and ψ) to Pol III′ forms Pol III*, which is about fivefold more processive than Pol III′ and is stimulated by single-stranded DNA-binding protein (8).

Holoenzyme includes all subunits found in Pol III*, plus the β subunit. Holoenzyme is the only form of Pol III that can efficiently replicate natural DNA template molecules in vitro in the presence of other replicative proteins (9, 24, 41, 43, 74). The holoenzyme is highly processive. It remains associated with templates in vitro for 30 to 40 min and may be sufficiently processive to replicate the entire *E. coli* chromosome without dissociation (22, 36, 74). To form a highly processive complex, holoenzyme must first combine with the primed template in an ATP-dependent reaction to form an initiation complex (71, 72). This complex is stable and is isolable by gel filtration (21, 72). Addition of the four required deoxynucleoside triphosphates results in the rapid replication of template DNA without dissociation of polymerase (9, 23). The ATP dependence of initiation complex formation presumably derives from the requirement for ATP in γδ-mediated transfer of β to a primed template (23, 47). Transfer of β can also be effected by τδ or τδ′ complexes in ATP-dependent reactions analogous to the γδ reaction (47).

It has been proposed that holoenzyme functions as an asymmetric, dimeric polymerase complex in which two functionally distinct polymerase halves are responsible for replication of either the leading or the lagging strand (23, 33, 39; reviewed in references 39 and 40). Functional specialization of the two polymerase halves could be used to solve the problem at the replication fork that results from continuous replication of the leading strand requiring a polymerase to remain bound throughout the entire cycle of replication and from discontinuous replication of the lagging strand requiring a polymerase to synthesize a 1,000- to 2,000-nucleotide Okazaki fragment, dissociate, and initiate synthesis of a new Okazaki fragment in about 1 s.

The asymmetric dimer hypothesis stemmed from experiments to examine the ATP dependence of holoenzyme activity, in which the ATP analog ATPγS was found to mediate formation of only one-half the amount of initiation complex as ATP and to cause dissociation of one half of the initiation complex formed with ATP (23). These results reveal a functional asymmetry between two halves of the dimer: one half can form initiation complexes by using ATPγS, whereas the other is sensitive to this analog.

Additional support for the asymmetric dimer hypothesis derives from examination of the relationship between the τ and γ subunits. Both subunits are products of *dnaX*; γ is produced following ribosomal frameshifting to a reading frame that contains a termination codon (3, 11, 67). Thus, the amino-terminal 47.5 kDa of τ and all but the last amino acid residue of γ are identical. The τ subunit, a DNA-dependent ATPase (30, 66), contains an additional 23.6-kDa carboxyl

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terminus that has sequence similarity to several proteins that interact with nucleic acids (38). That both subunits have ATP-binding sites (21, 30, 66) and are found within individual holoenzyme complexes (22) raises the possibility that these two subunits perform similar roles in different halves of holoenzyme. Consistent with this notion are data reported by O'Donnell and Studwell (47), who isolated minute quantities of δ and δ′ and showed that processive DNA synthesis, equivalent to that of holoenzyme, can be effected with a minimal combination of an αε complex, β, and γδ, τδ, or τδ′.

A rigorous test of the asymmetric dimer hypothesis requires the availability of large quantities of each holoenzyme subunit. As a step toward this goal, we have isolated the structural gene for δ. In this report, we describe the identification of the δ structural gene and its cloning, sequencing, analysis, and overproduction.

MATERIALS AND METHODS

Abbreviations. Abbreviations used are as follows: SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-D-galactopyranoside; HPLC, high-pressure liquid chromatography; and HIV, human immunodeficiency virus.

Chemicals. SDS, urea, *N,N′*-methylene-bisacrylamide, acrylamide, and Coomassie brilliant blue R-250 were obtained from Bio-Rad. [γ-^{32}P]ATP was purchased from ICN. Tris-HCl, bovine serum albumin, polyvinylpyrrolidone, dextran sulfate, and Ficoll were obtained from Sigma. Low-molecular-weight protein standards were purchased from Pharmacia. SeaKem LE agarose was purchased from FMC BioProducts. Bacteriophage λ DNA digested with *Hin*dIII was purchased from Promega and used as a double-stranded DNA molecular weight marker. All other chemicals were reagent grade.

Oligonucleotides. Oligonucleotides used in this work were synthesized by the University of Colorado Cancer Center Macromolecular Resources Core Facility. The two oligonucleotides used to reconstruct the 5′ end of the structural gene for δ were 5′-GATCTAGGAGGTAATAAATAATGATCCG CCTGTAC-3′ and 5′-AGGCGGATCATTATTTATTACCT CCTA-3′. The δ-gene-specific 51-mer probe was 5′-GCGGC GTATCTTTTACTTGGTAACGATCCTCTGTTATTGCAG GAAAGCCAG-3′.

Bacterial strains, plasmids, and media. MGC100, an isolate of RS320 [Δ(*lacIPOZYA*)*U169 Δlon araD139 strA supF*; gift of R. Sclafani, University of Colorado Health Sciences Center] that is resistant to a persistent phage that contaminates our fermentor (possibly bacteriophage T1), was used to isolate holoenzyme. Chromosomal DNA was purified from MAF102, a derivative of the wild-type strain MG1655 (14) into which *lexA3* and *uvrD* (70) had been introduced by P1 transduction. HB101 [*supE44 hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] (4) was used as the host strain for overexpression of the structural gene for δ. XL1Blue [F′ *proAB lacI*^{q}Z*ΔM15* Tn10 (Tet^r)/*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*], purchased from Stratagene, was the recipient strain in routine bacterial transformation experiments. pBlueScript II SK+ (Stratagene) and pUC19 (68, 75) were used as cloning vectors. pRT581 is a plasmid used to overproduce the HIV reverse transcriptase subunit p51 (62a). pJC1 is a plasmid used to overproduce the HIV nucleocapsid (76).

L broth (42) was used for routine growth of bacterial strains. Medium used in the δ-overexpression experiment contained 0.9% yeast extract, 0.8% peptone, and 0.9% potassium phosphate, pH 7.2. When required, ampicillin and tetracycline were used at 50 and 10 μg/ml, respectively.

Enzymes. Restriction enzymes and T4 DNA ligase (Promega), calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals), and T4 polynucleotide kinase (New England Biolabs) were used according to the manufacturers' instructions. The β subunit of holoenzyme was purified to homogeneity as described elsewhere (48) to a final concentration of 1.4 mg/ml.

Preparation of the δ subunit for amino acid sequence analysis. Holoenzyme was purified as described elsewhere (46) from 6 kg of MGC100 cells. Three preparations varying from 233 to 253 μg/ml and 5.4×10^5 to 7.0×10^5 U/mg were used.

Protein was concentrated by vacuum dialysis (0°C, 4 h) in a collodion bag (Schleicher & Schuell; molecular mass cutoff = 25,000 Da). The dialysis buffer was 63 mM Tris-HCl, pH 8.8, 10% glycerol, and 10 mM dithiothreitol. After removal of sample, the collodion bag was incubated in dialysis buffer for an additional 30 min to backwash the membrane. Three concentrated (8.5-mg/ml) holoenzyme samples (420, 508, and 508 μg) were prepared; SDS was added to each to a final concentration of 1%. Two samples (420 and 508 μg) were incubated for 5 min in a boiling water bath and loaded into two wells of a 1.5-mm SDS–7.5 to 17.5% polyacrylamide gel. For quantitation, 40 μg of purified β subunit was loaded onto each half of the gel. After electrophoresis for 16 h at 7 mA according to the method of Laemmli (28), one half of the gel, containing only the β-subunit standard, was stained with Coomassie brilliant blue R-250. The second half, containing holoenzyme and the β-subunit standard, was electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell; 0.45-μm pore size) (65) for 2 h at 0.5 A in transfer buffer (25 mM Tris-HCl, pH 8.2, 192 mM glycine, 20% methanol) by using a Hoefer Transblot apparatus. After transfer, the gel was stained with Coomassie brilliant blue R-250 to allow quantitative comparison to the half of the gel not transferred. The filter was rinsed in H_2O for 2 min, stained for 2 min (0.1% amino black 10B–10% acetic acid–45% methanol), destained for 3 min (10% acetic acid–45% methanol), and rinsed in water for 2 min (34).

The stained gels and the stained filter were scanned on a Molecular Dynamics computing densitometer model 300S and quantitated by using Molecular Dynamics ImageQuant version 3.0. The filter was then frozen while wet in a plastic, sealable bag and stored at −70°C until used for amino acid sequence analysis. Volumetric integration was used to estimate the amount of δ transferred onto the filter. Comparison of the β-subunit standard on the transferred and nontransferred gels indicated that about 75% of β, or 30 μg, had transferred out of the gel. Adsorption to the filter was assumed to be quantitative. Analysis of the filter showed that the mass of β in holoenzyme was 82% of the mass of β standard. Therefore, about 25 μg of β from holoenzyme had transferred to the membrane. In the two holoenzyme lanes, the mass of δ was calculated to be 70 and 80% of the mass of β. We conclude that about 18 and 22 μg (about 400 to 500 pmol) of δ was transferred to the filter.

Amino acid sequence determination. Approximately 40 μg of the δ subunit was cut out of two nitrocellulose membranes and digested in situ with trypsin. Tryptic fragments of δ were separated by using a narrow-bore Brownlee Aquapore Bu-300 column reversed-phase HPLC system. Amino-terminal sequence analysis of the peptides was performed on an Applied Biosystems 477A protein sequencer (1, 15).

Amino-terminal sequence analysis of intact δ subunit was

performed by the University of Colorado Cancer Center Protein Microsequencing Core Facility. Approximately 20 μg of the δ subunit (from the third sample of concentrated holoenzyme) was cut out of the ProBlott membrane (Applied Biosystems) and sequenced on an Applied Biosystems 477A protein sequencer equipped with an on-line PTH C-18 HPLC cartridge. Electrophoretic transfer and determination of transfer efficiency and mass of δ transferred were as described for the nitrocellulose membrane. After transfer, the membrane was rinsed in H_2O for 2 min, stained for 2 min (0.1% Coomassie brilliant blue R-250–50% methanol), destained for 3 min (10% acetic acid–50% methanol), and air dried (34).

Plasmid purification. Large-scale plasmid DNA isolation was performed by the alkaline-SDS lysis method (2). Plasmid DNA was further purified by processing through two CsCl-ethidium bromide equilibrium density gradients in a Sorvall T-127.0 rotor for 20 h at 187,800 × *g* or 40 h at 131,900 × *g*.

Isolation of high-molecular-weight genomic DNA. Cells were grown at 37°C to mid-exponential phase in L broth, pelleted by centrifugation, and resuspended in a solution of Tris-sucrose (10% sucrose, 50 mM Tris, pH 8.0). The suspension of cells was brought to 70 mM EDTA by addition of 0.25 M EDTA, pH 8.0, and incubated on ice for 5 min. Egg white lysozyme, RNase, and SDS were added to final concentrations of 0.5 mg/ml, 0.5 μg/ml, and 1%, respectively. A 2-h incubation on ice was followed by addition of a fresh solution of proteinase K to a final concentration of 100 μg/ml. The lysate was incubated at 37°C overnight, phenol-chloroform extracted five times, and processed through two 55% (wt/vol) CsCl equilibrium density gradients in a Sorvall T-127.0 rotor for 20 h at 187,800 × *g*. Ten-microliter aliquots of 1-ml fractions of the gradient were subjected to agarose gel electrophoresis and ethidium bromide staining to identify fractions containing DNA. Typically, DNA was found in two fractions.

Agarose gel electrophoresis. Horizontal agarose gels containing between 0.6 and 1.5% agarose in TBE (89 mM Tris base, 2.75 mM EDTA, 89 mM boric acid) were run in a Hoefer submarine gel apparatus. Gels used to separate chromosomal DNA restriction fragments (2 to 4 μg of DNA per lane) were run at 4°C for 16 to 24 h at 0.5 to 2 V/cm. Gels used for analysis of plasmid DNA were run at room temperature for 1 to 5 h at 2 to 10 V/cm. After electrophoresis, gels were stained for 10 min in a 0.8-μg/ml solution of ethidium bromide dissolved in TBE. Gels were illuminated with 254-nm UV light from a Fotodyne transilluminator and photographed with Polaroid type 667 film.

Gels used for purification of restriction fragments were made to contain between 0.6 and 1.0% agarose in TAE (40 mM Tris base, 20 mM acetic acid, 10 mM EDTA) and were run at room temperature at not more than 5 V/cm. Not more than 2 μg of DNA was loaded onto the gel in one wide well. The DNA fragment of interest was localized (without UV irradiation of the gel to avoid introducing potentially mutagenic, UV-induced lesions) by the following method. After electrophoresis, the gel was cut lengthwise to remove a thin gel slice containing molecular weight markers and a representative sample of the separated DNA. The gel slice was stained with ethidium bromide and UV irradiated to visualize the DNA fragments. A slice was made immediately below the DNA fragment of interest to mark its location. The remainder of the gel that had not been stained or irradiated was positioned next to the stained gel, and the DNA fragment of interest was localized. The fragment was excised from the unstained gel and purified by using the GeneClean II DNA purification kit from Bio 101. Chromosomal restriction fragments were purified by using an Elutrap apparatus (Schleicher & Schuell) according to the manufacturer's instructions.

Southern blotting. An agarose gel containing chromosomal DNA restriction fragments separated by electrophoresis was incubated for 10 to 15 min in 0.25 M HCl, rinsed briefly with deionized H_2O, and incubated for 20 min in denaturing solution (0.5 M NaOH, 1.5 M NaCl). A second, 30-min incubation in fresh denaturing solution was followed by a 30-min incubation in 10× SSC (1.5 M NaCl, 0.15 M Na citrate · $2H_2O$). DNA was transferred from the agarose gel to a GeneScreen nylon membrane (New England Nuclear) for 12 to 18 h by using a transfer buffer of 10× SSC and a conventional Southern blot assembly (59). After transfer, the membrane was incubated for 1 min in 0.4 M NaOH and neutralized for 1 min in 25 mM sodium phosphate, pH 6.0. The damp membrane was subjected to 1.6 kJ of UV light per m^2 from a germicidal lamp to cross-link the DNA to the membrane.

Preparation of 5′-end-labeled oligonucleotide. Fifty picomoles of the 51-mer oligonucleotide was 5′ end labeled with ^{32}P by incubation with 160 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol, 160 μCi/μl) and 10 U of T4 polynucleotide kinase in 50 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol) for 30 min at 37°C. Oligonucleotide was separated from nucleotide by using a 1-ml G-25 Sephadex (Pharmacia) gel filtration spin column equilibrated and developed with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mM NaCl. Purified oligonucleotide was frozen at −20°C until used in hybridization experiments.

Identification of chromosomal restriction fragments complementary to the 51-mer oligonucleotide. The GeneScreen membrane, to which restriction enzyme-digested, chromosomal DNA was transferred, was incubated in a sealable plastic bag containing 30 ml of prehybridization solution (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone [molecular weight = 40,000], 0.2% Ficoll 400,000, 0.1% sodium PP_i, 1.0% SDS, 10% dextran sulfate, 1 M NaCl, 50 mM Tris-HCl [pH 7.5], and 100 μg of low-molecular-weight, denatured salmon testis DNA per ml) for 10 to 20 h in a 65°C shaking water bath. Radioactive 51-mer oligonucleotide was added to the solution to yield 2×10^5 cpm/ml, and incubation was continued for 12 to 18 h. After hybridization, the membrane was washed by two 5-min incubations at room temperature in washing buffer (0.3 M NaCl, 60 mM Tris-HCl [pH 8.0], 2 mM EDTA), followed by two 30-min washes at 60°C in washing buffer supplemented with SDS to a final concentration of 1.0% and two 30-min washes with 0.1× washing buffer at room temperature. All washes were performed with constant agitation. The membrane was dried, wrapped in plastic wrap, and exposed for 4 to 48 h on a Molecular Dynamics Phosphorimager cassette containing a phosphor screen. The phosphor screen was scanned by using a Molecular Dynamics Phosphorimager model 400E, and the data were analyzed by using the ImageQuant version 3 program. Images were printed on a Hewlett-Packard LaserJet III modified to allow printing of 256 shades of gray.

Colony hybridization. The original clone of the δ gene was identified by colony hybridization (56). The radiolabeled 51-mer oligonucleotide was used as the probe.

DNA sequencing. The structural gene for δ was sequenced by Lark Sequencing Technologies, Inc., Houston, Tex., by the dideoxy chain termination method of Sanger et al. (57). The gene was sequenced in both directions.

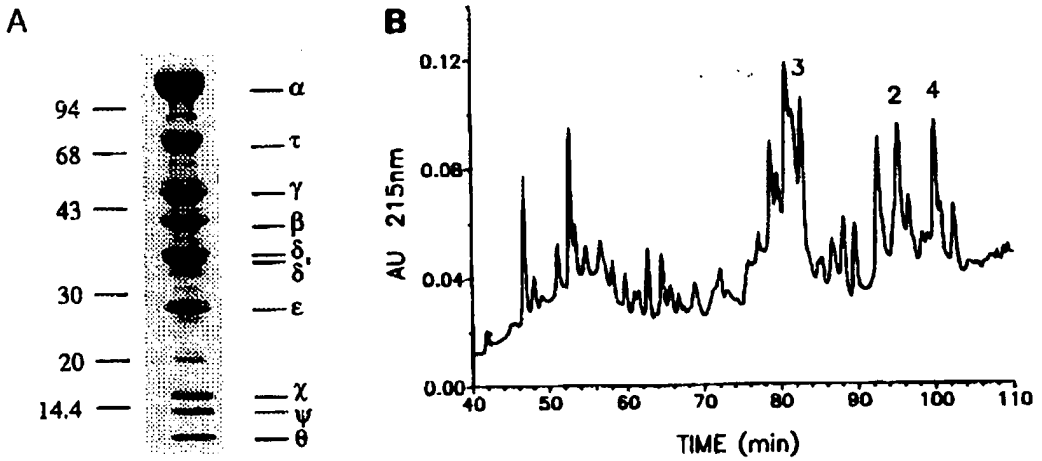

FIG. 1. Preparation of the δ subunit for amino acid sequence analysis. (A) Five hundred micrograms of holoenzyme was concentrated, resolved on an SDS–7.5 to 17.5% polyacrylamide gel, and transferred onto a nitrocellulose filter. The filter was stained and scanned by densitometry; the resulting computer-generated image is shown. The positions of molecular mass standards are shown in kilodaltons on the left; the holoenzyme subunits are labeled on the right. (B) The δ subunit was cut out of the blot shown in panel A and digested directly on the filter with trypsin. The resulting peptides were separated by reversed-phase HPLC. Peptides corresponding to the peaks immediately below the numeric labels were subjected to amino-terminal sequencing.

Overexpression of the δ subunit. Cells from a fresh overnight liquid culture were diluted 1:100 in 25 ml of fresh broth and grown at 37°C, with vigorous shaking, to an A_{600} of 0.4. IPTG was added to 10 ml of each culture to a final concentration of 1 mM, and growth was monitored by measurement of the A_{600} at 30-min intervals. After 4 h, 1 ml of cells was pelleted by centrifugation for 5 min, resuspended in 100 μl of lysis buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5% SDS, 100 mM β-mercaptoethanol, 15% glycerol, 0.02% bromphenol blue) and incubated in a boiling water bath for 10 min. Following a second, 2-min centrifugation to remove cell debris, the cell lysate was incubated in a boiling water bath for 5 min, and a portion representing 0.2 A_{600} unit of cells was loaded onto an SDS–7.5 to 17.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and scanned on a Molecular Dynamics scanning densitometer, model 300S, to quantitate the overproduction of the δ subunit.

Computer analysis. GenBank (version 67) was searched by using the TFASTA program that translated the GenBank DNA sequence in all six reading frames and performed a similarity search by the Pearson and Lipman method (51). Protein translation, inverted repeat analysis, and codon usage analysis were performed by using PC/GENE (Intelligenetics) running on an IBM personal computer.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number of the sequence reported in this paper is M94267.

RESULTS

To identify and clone the structural gene for δ, we used a reverse genetic approach in which terminal and internal peptide sequences for δ were obtained and used to design an oligonucleotide hybridization probe to isolate the gene.

Amino acid sequences of δ peptides. Isolation of peptides in adequate quantity for sequence analysis required preparation of a highly concentrated sample of holoenzyme, so that 10 to 20 μg of δ protein could be separated from other holoenzyme subunits from each lane of an SDS-polyacrylamide gel. Roughly 500 μg of holoenzyme was applied to each lane of a gel (Fig. 1A) and electrophoretically transferred onto a ProBlott membrane or nitrocellulose membrane. Densitometry of the filters and gels indicated that 18 to 22 μg of δ had been transferred from each lane.

The δ subunit was excised from the ProBlott membrane and subjected to amino-terminal sequencing as described in Materials and Methods. Figure 2A shows the 21-amino-acid sequence that was obtained (sequence 1). Four amino acid residues (designated X) within the 21-amino-acid sequence were not identifiable.

To obtain internal amino acid sequences of the δ protein, fragments of δ were produced by tryptic digestion of δ. The fragments were separated on a microbore HPLC column, and three well-resolved peptides (Fig. 1B) were selected for amino-terminal sequencing to obtain three internal amino acid sequences (Fig. 1A, sequences 2, 3, and 4). The first three residues of sequence 2 overlapped the amino-terminal sequence of δ by 3 amino acid residues (Fig. 2A, underlined residues).

Data base searches using the four δ peptide sequences. Sequence identity searches were performed between each of the four δ peptide sequences and DNA sequences in GenBank (version 67) translated in all six reading frames. A partial match to sequence 1 and a complete match to sequence 2 were found to amino acids coded by 110 bp of DNA sequence reported downstream of the *rlpB* gene sequence (62). No matches to sequence 3 or 4 were found. The significance of the matches was unclear since they occurred in two different reading frames and contained mismatches and omissions relative to sequence 1.

The sequence that contained the imperfect but highly similar match was downstream of the *rlpB* gene (62) and thus might not have been subjected to the same scrutiny as *rlpB* sequences. Although the mismatches were likely due to errors in the DNA sequence, we decided to isolate the gene from a wild-type *E. coli* K-12 strain and examine whether the DNA sequence exactly matched all of our δ protein se-

A

#1 M I X L Y P E Q L X A Q L N X G L X A A Y
#2 A A Y L L L G N D P L L L Q E S Q D A V
#3 L S L L W P D G
#4 V E Q A V N D A A H F T P F

B

701..GGTAACTGATGATTCGGTTGTACCCGGAACAACCTGCTACGCTCAATGAA

RF1 - L N I R L Y P E Q P A T L N E
RF2 G N - -
#1 M I X L Y P E Q L X A Q L N X

751..GGGCTCGGCCGCGGCGTATCTTTTACTTGGTAACGATCCTCTGTTATTGC

RF1 G L G R
RF2 A A Y L L L G N D P L L L Q
#1 G L X A A Y
#2 A A Y L L L G N D P L L L Q

801..AGGAAAGCCAGGACGCTGTTCGTCAGGTAGCTGCGGCACAAGGATTCGAA

RF2 E S Q D A V R Q V A A Q G F E E
#2 E S Q D A V

FIG. 2. Peptides of the δ subunit. (A) Sequence 1 is the amino-terminal sequence of δ. Sequences 2, 3, and 4 were obtained from purified tryptic fragments of δ. The first three residues of sequence 2 and the last three residues of sequence 1 are identical (underlined). "X" indicates undetermined residues. (B) DNA sequence and base numbering are as reported elsewhere (62). The two adjacent termination codons of *rlpB* are underlined. RF1 and RF2 indicate protein sequences predicted from two reading frames of the DNA. Sequences 1 and 2, as presented in panel A, are indicated by "#1" and "#2". Matches between sequences 1 and 2 and amino acids predicted from the DNA sequence are indicated by colons.

quence information. We selected a 51-nucleotide stretch (see Materials and Methods) that, when translated, corresponded perfectly to the first 17 amino acids of sequence 2, thus minimizing the likelihood that the oligonucleotide contained errors relative to the true sequence of the chromosome. The oligonucleotide is specific to DNA near the 5′ end of the predicted gene encoding δ.

Physical mapping of the structural gene for δ. To define a restriction map of the region of the chromosome containing the structural gene for δ, MAF102 chromosomal DNA was digested with all single- and double-enzyme combinations of *Eco*RI, *Eco*RV, *Bam*HI, *Bgl*II, *Hin*dIII, and *Pst*I. The digested DNA samples were transferred to a GeneScreen nylon membrane and hybridized with the δ-gene-specific, radiolabeled, 51-mer oligonucleotide. Autoradiography revealed the sizes of restriction fragments complementary to the oligonucleotide (Fig. 3A). From the blot (Fig. 3A), a restriction map (Fig. 3B) of the region of the chromosome containing the structural gene for δ was constructed and used to identify a restriction fragment that contained the entire structural gene for δ. The oligonucleotide probe hybridized to a 3.6-kb *Bgl*II fragment (Fig. 3B), indicating that this fragment contained at least the 5′ end of the gene. Digestion with *Eco*RV and *Bgl*II produced a 1.6-kb restriction fragment (Fig. 3B) complementary to the oligonucleotide used as a probe. These restriction fragment sizes plus others were consistent with the restriction map reported by Kohara et al. (25) (discussed below). The gene *rlpB*, which is

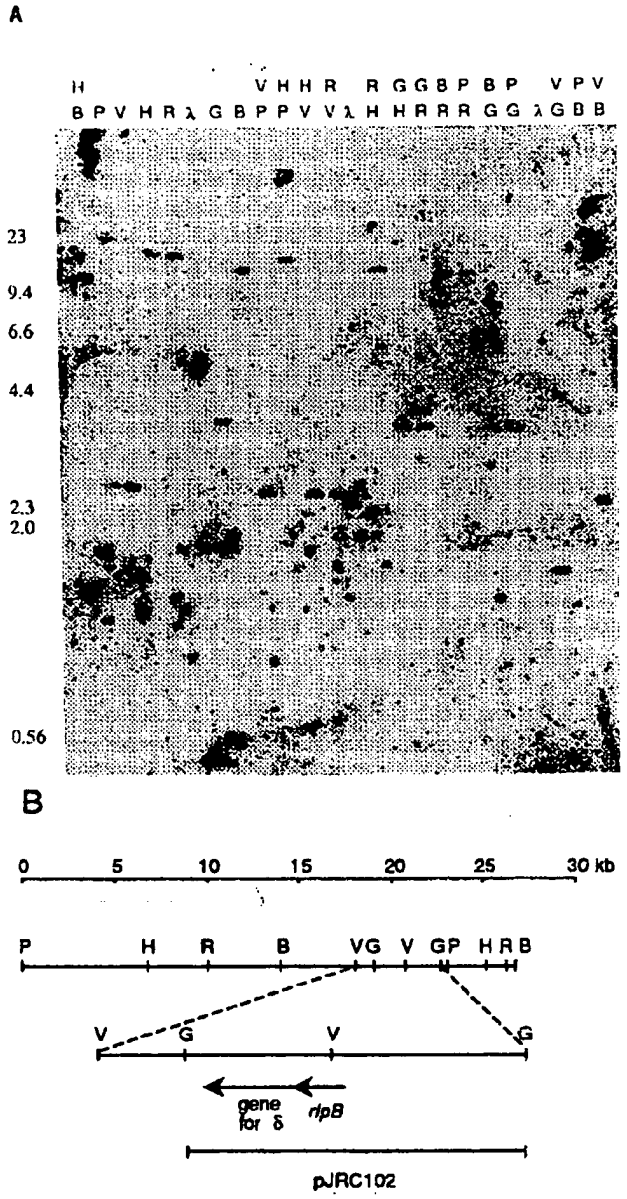

FIG. 3. Restriction map of the region of the chromosome containing the δ-subunit structural gene. (A) MAF102 chromosomal DNA was blotted onto GeneScreen nylon membrane and hybridized with the 51-mer oligonucleotide specific for the δ structural gene. Restriction enzyme abbreviations: B, *Bam*HI; G, *Bgl*II; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; V, *Eco*RV. Size standards expressed as kilobases are indicated to the left. (B) A restriction map of the region of the chromosome containing the structural gene for δ was constructed on the basis of the restriction digestion patterns observed in the Southern blot. The 3.6-kb *Bgl*II fragment that was cloned into pBlueScript II SK+ to generate pJRC102 is enlarged to show the structural gene for δ and the gene *rlpB*.

immediately upstream of the DNA complementary to the oligonucleotide, contains an *Eco*RV site 239 bp from the putative initiation codon of the δ structural gene (62). Therefore, we concluded that ca. 1,350 bp of the *Eco*RV-*Bgl*II fragment was available to encode δ. The molecular mass of δ is estimated from SDS-polyacrylamide gel electrophoresis to be about 34,000 Da. Thus, δ contains about 310 amino acid residues. An open reading frame containing 310 codons, or about 1 kb, would be required to encode δ. We concluded that the entire gene exists within the first 1,350 bp downstream of *rlpB* and is contained on the 1.6-kb *Eco*RV-*Bgl*II fragment and on the 3.6-kb *Bgl*II fragment.

The restriction map in Fig. 3B was also used to determine the chromosomal map location of the structural gene for δ. Comparison of the map in Fig. 3B to the complete restriction map of the *E. coli* chromosome (25) revealed a single region corresponding to ca. 15 min, the map position reported for *rlpB* (62); this is consistent with the δ structural gene mapping downstream of *rlpB*. *rlpB* is part of the 15-kb cluster of genes including, in order, *leuS* (leucyl-tRNA synthetase), *rlpB*, *mrdA* (peptidoglycan synthetase), *mrdB*, *rlpA* (rare lipoprotein), and *dacA* (D-alanine carboxypeptidase) (19, 20, 62). The map position of the δ structural gene was more precisely determined by using the most recent *E. coli* restriction map, in which the physical and genetic maps of *E. coli* are correlated (53, 54). In this map, *rlpB* is located at 14.6 centisomes, or 682.5 kb. (The centisome is defined as 1% of the chromosome, or 47,736 bp, and is used in place of "minute" to avoid problems arising from the noncolinear relationship between the physical and genetic maps [53, 54]). *rlpB* (525 bp) is immediately upstream of the gene encoding δ and is transcribed counterclockwise (62). Therefore, the structural gene for δ begins at 682 kb and ends at 681 kb of the *E. coli* chromosome.

Cloning of the structural gene for δ. Having tentatively identified the structural gene for δ, we proceeded to clone it and define its sequence to determine whether the mismatches (Fig. 2B) were due to errors in the DNA or protein sequence. MAF102 chromosomal DNA was digested with *Bgl*II, and DNA in the size range of 3.6 kb was purified. This DNA was cloned into the *Bam*HI site of pBlueScript II SK+ and transformed into XL1Blue. Six hundred ampicillin-resistant colonies were screened by colony hybridization (56), using the 51-mer oligonucleotide 5′ end labeled with ^{32}P to identify clones of the structural gene for δ. Three positive colonies were identified. Plasmid DNA from these three colonies was characterized by *Sal*I restriction enzyme analysis. In addition to the *Sal*I site of pBlueScript II SK+, two *Sal*I sites, separated by 286 bp, were predicted to be in the 3.6-kb *Bgl*II fragment (62). *Sal*I restriction analysis revealed the presence of this 286-bp fragment in plasmids obtained from all three colonies. Moreover, the identity of the *Sal*I restriction maps of all three plasmids indicated that the same 3.6-kb *Bgl*II fragment had been ligated into pBlueScript II SK+ in all three isolates (data not shown). One of these three plasmids, pJRC102, was chosen for further study.

DNA sequence analysis of the structural gene for δ. The structural gene for δ contained in pJRC102 was subjected to dideoxy chain termination sequencing (57). The primer used for the first sequencing reaction was a 17-mer complementary to DNA 89 bp upstream of the predicted initiation codon of the δ structural gene (62), i.e., within the *rlpB* gene. Choosing to prime within the rigorously sequenced *rlpB* gene provided confidence that the DNA sequence used to design the first primer was error free. The DNA sequence obtained with the first primer was used to design primers for additional sequencing reactions. This strategy (Fig. 4) was used repeatedly to gain double-strand sequence data for the entire open reading frame (Fig. 5).

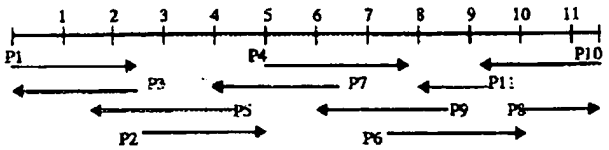

FIG. 4. Strategy for sequencing the structural gene for δ. The structural gene is depicted as the top horizontal line and is divided into increments of 100 bp. Arrows indicate the extent and direction of sequencing performed by each of 11 primers (P1 to P11). All of the gene was sequenced in both directions.

Compared to our sequence, the previously reported 230-bp DNA sequence downstream of *rlpB* (62) is missing 3 bases and contains 1 extra base and 6 mismatched bases. Our limited sequence data of the 3′ end of *rlpB* are in complete agreement with the sequence previously reported (62). The sequence presented in Fig. 5 contains one 1,029-base open reading frame, or 343 codons, and is predicted to encode a protein of 38,703 Da. This is in reasonable agreement with the size of δ, 34,000 Da, estimated by SDS-polyacrylamide gel electrophoresis. The identified open reading frame encodes all four partial amino acid sequences reported in Fig. 1.

The open reading frame contains an AUG initiation codon that overlaps the two adjacent termination codons of *rlpB* and that follows a potential weak ribosome-binding site (58) (Fig. 5). This site is 6 bases upstream of the initiation codon and is predicted to pair with the 16S rRNA through two G-C base pairs and one A-U base pair, thus satisfying the spacing and minimal base pairing requirements of natural ribosome-binding sites (60). No consensus σ^{70} promoter was identified upstream of the gene encoding δ. These three genetic characteristics, overlap of initiation and termination codons, a weak ribosome-binding site, and absence of a promoter, suggest that the primary mechanism of expression of δ is through translational reinitiation following translation of *rlpB* (13). Thus, *rlpB* and the gene encoding δ may be part of the same operon. The possibility that a gene downstream of the gene encoding δ is part of this putative operon exists. An AUG codon exists only 1 bp after the termination codon of the gene for δ, and no termination codon exists in the 18 codons downstream of this AUG.

A striking feature of the DNA sequence is an inverted repeat (Fig. 6) beginning 24 bases downstream of the initiation codon of the gene encoding δ, i.e., within the gene. Transcription through this inverted repeat would produce mRNA with the potential to form a hairpin structure (calculated $\Delta G = -27.8$ kcal/mol [ca. −116 kJ/mol] [63]). The hairpin is followed by a uridine-rich region, including four uridine residues in a row (Fig. 6). These two features, a hairpin structure followed by a string of uridine residues, are the hallmarks of a classical rho-independent transcription terminator (52). If biologically active, this terminator would provide a mechanism to attenuate expression of δ.

We analyzed the codon usage of the gene encoding δ (Table 1) to determine the percentage of infrequently used codons in the gene. The set of eight rare codons (AUA, UCG, CCU, CCC, ACG, CAA, AAU, and AGG) was compiled from a survey of 25 nonregulatory genes in *E. coli* (26); these codons occur at a combined average of 3.5% in the reading frames of these genes. The subset of 10 ribosome

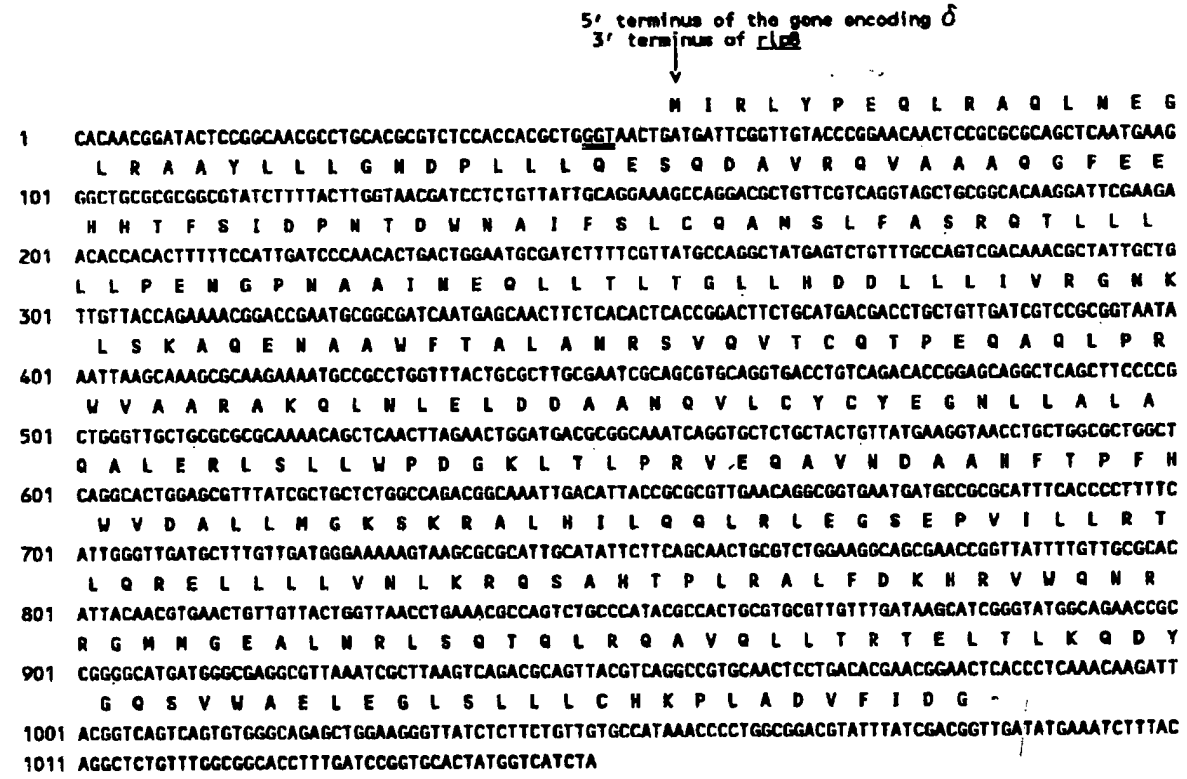

FIG. 5. DNA sequence of the structural gene for the δ subunit. The arrow indicates the 3′ end of *ripB* and the 5′ end of the structural gene for δ. The termination codon(s) of *rlpB* begins at nucleotide 56, and the δ gene initiation codon begins at nucleotide 55. Double underlining indicates a potential Shine-Dalgarno site. Amino acids are represented in single-letter code above the first base of each codon. The dash indicates the translational termination codon for the gene encoding δ.

protein genes, which are very highly expressed, has an average of 1.7% rare codons. This can be compared to the presumably random occurrence of rare codons in noncoding frames, an average of 11.7% (26). Konisberg and Godson have hypothesized that rare codon usage is partially responsible for the low expression of the replication gene *dnaG* (26). The gene encoding δ contains 8.7% rare codons (Table 2). Comparison of this value to values for six other *E. coli* genes (Table 2) indicates a strong bias of *dnaG*, *rlpB*, and the structural gene for δ toward use of rare codons, which could contribute to modulation of expression of these three genes. Rare codons occur with decreasing frequency in the four holoenzyme-subunit genes *dnaQ*, *dnaX*, *dnaE*, and *dnaN*.

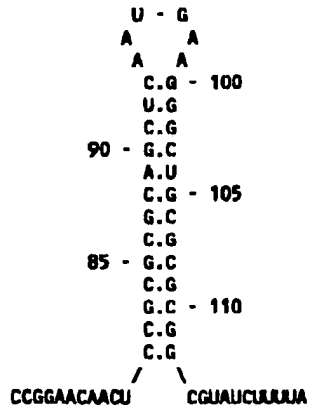

FIG. 6. A potential transcription terminator in the δ mRNA transcript. The hairpin structure, which has a calculated ΔG of −27.8 kcal/mol (ca. −116 kJ/mol), extends from bases 81 to 112 of the reported DNA sequence (Fig. 5). The stem contains 11 GC base pairs, 1 AU base pair, and 1 GU base pair and is followed on the 3′ end by a region rich in uridine residues, including a run of four uridines in a row. Numbers correspond to the base numbering of Fig. 5.

Amino acid sequence analysis of δ. Translation of the DNA sequence indicates that δ is a protein of 38,703 Da. The primary sequence shows a preponderance of leucine residues, which often occur in stretches of 2 to 5 residues and which compose 20% of the protein. A search of the sequence for a consensus leucine zipper motif, which is characterized by 4 or 5 leucine residues spaced by 7 amino acid residues (29, 50), revealed none, even if functional amino acid substitutions (18) are allowed in place of the characteristic leucine residues. Further inspection of the sequence revealed no matches to other common protein motifs, e.g., zinc finger or helix-turn-helix. Additionally, no significant primary sequence similarity was observed between δ and the gene 44 or gene 62 product of phage T4. The function of the gene 44-gene 62 protein complex in T4 replication is analogous to that of the γδ complex; both complexes load their cognate polymerase clamps onto primed templates.

The γδ complex of holoenzyme is a DNA-dependent ATPase (49). Analysis of the primary sequence of δ revealed a region of the protein that resembles the Walker A-consen-

TABLE 1. Codon usage within the gene encoding δ

Codon	Amino acid	No. of times used	% of total codons
UUU	Phe	7	2.0
UUC	Phe	2	0.6
Total	Phe	9	2.6
UUA	Leu	14	4.0
UUG	Leu	14	4.0
CUU	Leu	8	2.3
CUC	Leu	10	2.9
CUA	Leu	1	0.2
CUG	Leu	23	6.7
Total	Leu	70	20.4
AUU	Ile	4	1.1
AUC	Ile	4	1.1
AUA[a]	Ile	0	0
Total	Ile	8	2.3
AUG	Met	5	1.4
GUU	Val	6	1.7
GUC	Val	1	0.2
GUA	Val	3	0.8
GUG	Val	6	1.7
Total	Val	16	4.7
UCU	Ser	2	0.5
UCC	Ser	1	0.2
UCA	Ser	1	0.2
UCG[a]	Ser	2	0.5
AGU	Ser	4	1.1
AGC	Ser	4	1.1
Total	Ser	14	4.1
CCU[a]	Pro	2	0.5
CCC[a]	Pro	3	0.8
CCA	Pro	3	0.8
CCG	Pro	5	1.4
Total	Pro	13	3.8
ACU	Thr	3	0.8
ACA	Thr	5	1.4
ACG[a]	Thr	4	1.1
ACC	Thr	4	1.1
Total	Thr	16	4.7
GCU	Ala	7	2.0
GCC	Ala	6	1.7
GCA	Ala	6	1.7
GCG	Ala	18	5.2
Total	Ala	37	10.8
UAU	Tyr	2	0.8
UAC	Tyr	3	0.8
Total	Tyr	5	1.5
CAU	His	7	2.0
CAC	His	2	0.5
Total	His	9	2.6
CAA[a]	Gln	9	2.6
CAG	Gln	20	5.8
Total	Gln	29	8.5
AAU[a]	Asn	10	2.9
AAC	Asn	7	2.0
Total	Asn	17	5.0
AAA	Lys	8	2.3
AAG	Lys	2	0.5
Total	Lys	10	2.9
GAU	Asp	7	2.0
GAC	Asp	8	2.3
Total	Asp	15	4.4
GAA	Glu	15	4.3
GAG	Glu	5	1.4
Total	Glu	20	5.8
UGU	Cys	2	0.5
UGC	Cys	3	0.8
Total	Cys	5	1.5
UGG	Trp	7	2.0
CGU	Arg	6	1.7
CGC	Arg	12	3.4
CGA	Arg	2	0.5
CGG	Arg	3	0.8
AGA	Arg	0	0
AGG[a]	Arg	0	0
Total	Arg	23	6.7
GGU	Gly	5	1.4
GGC	Gly	4	1.1
GGA	Gly	4	1.1
GGG	Gly	2	0.5
Total	Gly	15	4.4

[a] Rare codons (26).

sus ATP-binding site, (Ala/Gly-1)–(X-2–X-3–X-4–X-5)–Gly-6–Lys-7–(Ser/Thr-8) (16, 44, 69). The sequence in δ between amino acid residues 219 and 225 is Ala-1–(Leu-2–Leu-3–Met-4)–Gly-6–Lys-7–Ser-8. A major deviation of this sequence from the consensus is the existence of 3 residues instead of 4 between Ala-1 and Gly-6. We know of no example of a characterized ATP-binding site with this deviation from the consensus. In addition, 35 of 37 ATP-binding sites (compiled from references 16, 17, 44, and 69) contain a Gly at position X-4. The two exceptions, RecA and DnaB, contain a Ser residue at this position. Finally, whereas ATP-binding sites form a loop between a β-sheet and an α-helix (44), two different secondary structure predictions for δ (6, 12) depict the putative ATP-binding site as being within a region rich in α-helical structure and devoid of β-sheets (data not shown).

Construction of a δ-overproducing plasmid. As a final test that the structural gene for δ had been isolated, we placed it in an expression vector to determine whether the gene directed synthesis of a protein the size of δ. The overexpression vector, which contains the strong *tac* promoter, has been used to overproduce several holoenzyme subunits and HIV proteins. Our strategy involved cloning all but the first 13 bases of the δ open reading frame into pRT581 (a plasmid that overproduces the p51 subunit of HIV reverse transcriptase [62a]), removing the p51 open reading frame, and reconstructing the 5′ end of the δ structural gene with synthetic oligonucleotide designed to replace rare codons with more commonly used synonymic codons (Fig. 7).

TABLE 2. Percentage of rare codons[a] in the three reading frames of seven *E. coli* genes

Gene[b]	% in:		
	Frame 1[c]	Frame 2	Frame 3
Gene encoding δ	8.7	11.4	8.7
rlpB	9.8	13.4	6.2
dnaE	4.9	13.4	10.8
dnaN	4.1	12.5	11.4
dnaX	6.8	15.6	6.7
dnaQ	7.0	13.2	11.5
dnaG	11.3	12.4	12.9

[a] Rare codons include AUA (Ile), UCG (Ser), CCU and CCC (Pro), ACG (Thr), CAA (Gln), AAU (Asn), and AGG (Arg) (26).
[b] Sequence data were derived from the following sources: *dnaG* (26), *rlpB* (62), *dnaE* (64), *dnaN* (48), *dnaX* (10), and *dnaQ* (32).
[c] Frame 1 = coding frame.

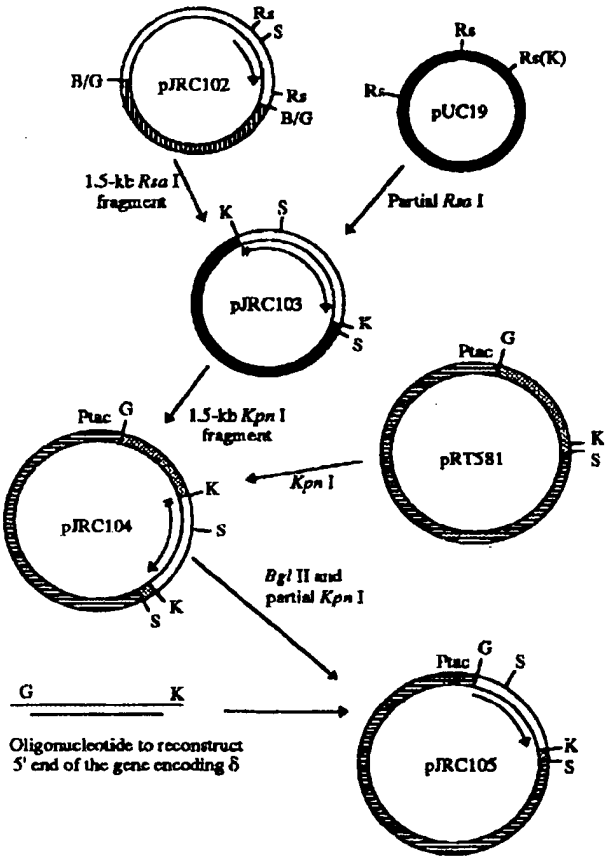

FIG. 7. Construction of a plasmid that overexpresses δ. pJRC102 contains the 3.6-kb *Bgl*II δ-gene-containing fragment of MAF102 chromosomal DNA, cloned into the *Bam*HI site of pBlueScript II SK+. The plasmid was digested with *Rsa*I. A 1.5-kb fragment, containing all but the first 10 nucleotides of coding region, was cloned into pUC19 at the *Rsa*I site which is internal to the unique *Kpn*I site of pUC19, generating pJRC103. The 1.5-kb fragment of pJRC103 was cloned into the unique *Kpn*I site of pRT581. The resulting plasmid, pJRC104, was digested with *Bgl*II and *Kpn*I to remove all but the last 14 codons of the p51 open reading frame. Cloned into *Bgl*II- and *Kpn*I-digested pJRC104 was a synthetic, duplex oligonucleotide which served to reconstruct the 5′ end of *holA* and provide a consensus Shine-Dalgarno site, an A/T region between the initiation codon and the Shine-Dalgarno site, and an adenosine residue 3 nucleotides 5′ to the initiation codon. The coding strand of the duplex oligonucleotide is 5′-GATCTAGGAG GTAATAAATAATGATCCGCCTGTAC-3′. The product of this cloning, pJRC105, was the plasmid used to overexpress δ. Restriction site abbreviations: G, *Bgl*II; K, *Kpn*I; Rs, *Rsa*I; S, *Sal*I. The curved arrow represents the gene encoding δ. Two short parallel lines at the beginning of the arrow indicate truncation of the gene caused by digestion with *Rsa*I.

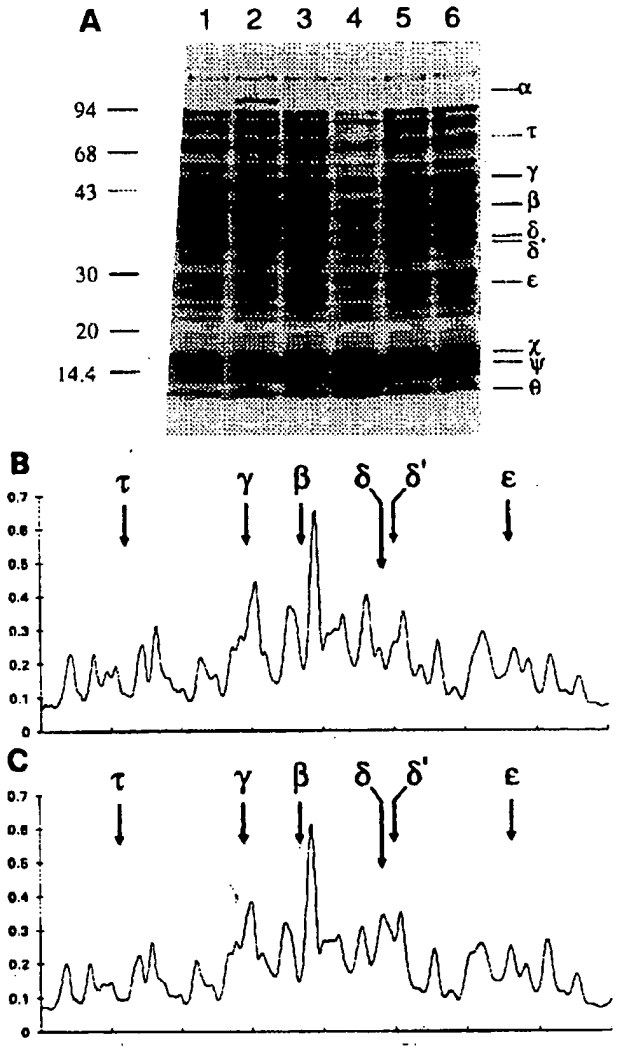

FIG. 8. Overexpression of the δ subunit. (A) Cells were grown to an A_{600} of 0.4, induced with IPTG for 4 h, and lysed as described in Materials and Methods. Proteins of the cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lysates from equal masses of cells were added to each lane of the gel. Lane 1, HB101; lane 2, HB101, IPTG induced; lane 3, HB101 containing pJC1, the HIV nucleocapsid protein overproducer; lane 4, HB101 containing pJC1, IPTG induced; lane 5, JRC107, which contains the δ overexpression plasmid pJRC105; lane 6, JRC107, IPTG induced. The migration of purified holoenzyme subunits is indicated on the right; the positions of molecular mass markers, in kilodaltons, are on the left. (B and C) Densitometric scans of lanes 5 and 6, respectively. The origin of the lanes is to the left, and the arrows indicate the locations of holoenzyme subunits.

Overexpression of δ. The candidate δ expression plasmid (pJRC105, Fig. 5) was transformed into HB101 to generate strain JRC107, which was used to test whether the isolated gene could produce a protein the size of the δ subunit. Cells were induced by treatment with IPTG. In JRC107, IPTG-dependent production of a protein that comigrates with the δ subunit from purified holoenzyme was detected (Fig. 8A, compare lanes 5 and 6). HB101 was used as a control to ensure that no protein the size of δ is produced upon IPTG induction of a strain lacking plasmid (Fig. 8A, lanes 1 and 2). HB101 containing pJC1, which overproduces HIV nucleocapsid from the same vector used to construct pJRC107 (76), was tested as another control; IPTG induction of this strain causes production of the 6,380-Da nucleocapsid protein and no protein the size of δ (Fig. 8A, lanes 3 and 4). We conclude

that overproduction of a protein the size of δ occurs only in IPTG-induced cells carrying a plasmid with the gene predicted to encode δ. On the basis of densitometric scans of lane 5 (noninduced JRC107) and lane 6 (IPTG-induced JRC107) (Fig. 8B and C, respectively), we calculate that the δ subunit represents approximately 4% of the total soluble protein of IPTG-induced JRC107.

DISCUSSION

The primary goal of this work was to isolate and characterize the structural gene for the δ subunit of DNA polymerase III holoenzyme. We took an approach to cloning the structural gene for δ in which we determined the amino-terminal and several internal sequences for δ and used this information to devise a strategy to map the gene and to screen for clones of it.

A preliminary search of sequence data bases revealed strong but imperfect matches between two of the four sequences obtained for δ in a 110-bp stretch of DNA downstream of *rlpB*. Because of the predicted mismatches and deletions relative to the amino-terminal sequence of δ and because two different reading frames were required in order to align the sequences, we isolated the candidate gene from a wild-type *E. coli* K-12 strain to determine whether the mismatches were due to sequencing errors or whether the sequence identified in the data base was from an inert pseudogene.

For a hybridization probe, we synthesized a 51-mer oligonucleotide based on δ sequences that matched an amino acid sequence that agreed with both experimentally obtained δ sequences and sequences identified in the data base. This sequence hybridized to a unique set of restriction fragments generated from the *E. coli* chromosome. A map generated from these restriction digestions was consistent with the region of the *E. coli* chromosome downstream of *rlpB*, the originally identified locus for the δ structural gene. We cloned this region and sequenced it to determine whether the imperfect alignment was due to errors in the reported DNA sequence.

The 1,029-bp open reading frame identified from the sequence aligned perfectly in one reading frame with all four δ protein sequences and encoded a protein of 38,703 Da, consistent with the expected size of δ. That the isolated candidate gene aligned with protein sequences not contained in the data base and not contained in our probe added confidence that we had identified the authentic structural gene for δ. An overproducing plasmid constructed to express δ using the *tac* promoter, an optimal ribosome-binding site, and additional sequence features included to allow high-level gene expression directed synthesis of a protein that comigrated with the δ subunit of holoenzyme. Preliminary data (5a) indicate that the candidate overproducing strain expresses δ functional activity, based on its ability to reconstitute holoenzyme activity at least 50-fold over a control strain. Together, these data indicate that we have isolated the structural gene for δ, a key step in the study of holoenzyme activity and of the regulation of holoenzyme gene expression. The O'Donnell laboratory has independently cloned the structural gene for δ (7a). As suggested by K. Marians (Sloan Kettering), the O'Donnell laboratory and our laboratory have agreed to designate the gene *holA*.

Sequence analysis revealed that the initiation codon of *holA* overlaps the two adjacent termination codons of *rlpB*. Although a weak Shine-Dalgarno sequence can be identified 6 bases upstream of the *holA* initiation codon, the closest consensus σ^{70} promoter upstream of *holA* is the *rlpB* promoter. Therefore, it is likely that *holA* is transcribed as part of a polycistronic message initiating at the *rlpB* promoter. The presence of an AUG start codon 1 bp downstream of *holA* followed by 18 nontermination codons raises the possibility that at least one additional gene is transcribed from the *rlpB* promoter. The proximity of the *holA* Shine-Dalgarno site to the *rlpB* termination codon(s) (4 bp) and the overlap of termination and initiation codons suggest that translational reinitiation, following translation of *rlpB* mRNA, could be involved in expression of *holA* (13). A genetic organization of this kind is typical of genes coordinately controlled as part of one operon.

A second mechanism of regulation of δ expression could occur at the level of transcription termination. We find a classical rho-independent transcription terminator located shortly after the initiation codon of *holA*. Since transcription and translation are coupled in procaryotes, translation of *holA* mRNA, initiated by translational reinitiation, would inactivate the transcription terminator. However, when translation does not initiate immediately, transcription would produce an mRNA that is able to adopt the strong hairpin structure, and transcription termination would occur. By this mechanism, differential production of RlpB and δ could be effected.

A further level of regulation might occur at the level of codon usage. The frequency of rare codons in *holA*, 8.7%, is 2.5-fold higher than that in nonregulatory genes and 5-fold higher than that in highly expressed ribosome subunit genes. Thus, a bias toward rare-codon usage might contribute to the low level of δ production. As a component of holoenzyme, δ is predicted to have a copy number of 10 to 20 molecules per cell. However, caution should be used in attributing a major effect on gene expression to rare-codon usage. Although codon usage bias probably has some effect, it is most likely of secondary importance to features such as the strengths of promoter or Shine-Dalgarno sites (7) or possible regulation of the *rlpB* promoter.

As mentioned, *rlpB*, the gene encoding a rare lipoprotein, and *holA* may exist in one operon. It is also possible that one or more of the remaining genes in the *leuS-dacA* region of the chromosome are in this putative operon. By being in the same operon, *holA* and *rlpB* would be subject to, for example, a modulator of transcription originating from the *rlpB* promoter. The possibility that *holA* and *rlpB* are coregulated might be a clue to the more general possibility that the two macromolecular synthesis systems, chromosomal replication and cell envelope biogenesis, are coordinately regulated; the putative operon containing *rlpB* and *holA* would contain genes involved in at least these two distinct macromolecular biosynthetic processes. A link between replication and cell envelope biogenesis proteins has been reported by Sakka et al. (55), who showed that the catalytic α subunit of holoenzyme negatively regulates expression of signal peptidase II, the enzyme that cleaves the leader peptide from prolipoproteins, a requisite step for lipoprotein insertion into the membrane. Thus, one subunit of holoenzyme, α, is apparently involved in the regulation of the signal peptidase II that processes the product of the gene upstream of (and possibly coregulated with) *holA* to a biologically active form. Whether this leads to a relevant genetic circuit remains unknown. Although grouping of genes whose products are functionally dissimilar is not common, at least two major examples exist in *E. coli*. The first, the macromolecular synthesis operon, encodes the *dnaG* (primase), *rpoD* (σ^{70} subunit of RNA polymerase), and *rpsU* (S21 ribosome

subunit) genes (5, 31), and the second, the macromolecular synthesis II operon, encodes *dnaE* (α polymerase subunit of holoenzyme), *cdsA* (CDP-diglyceride synthetase), *lpxA* (UDP-*N*-acetylglucosamine acetyltransferase), and *lpxB* (lipid A disaccharide synthase) (63a).

The δ subunit, as part of the γδ complex, is required to load the β subunit onto a primed template in a reaction that requires ATP hydrolysis. In a study to determine which components of the γδ complex (γ, δ, δ′, χ, and ψ) hydrolyze ATP, Onrust et al. (49) found that neither γ nor δ alone is a DNA-dependent ATPase but that the two subunits combined have significant ATPase activity. Given that γ binds ATP (66) and that τ, an alternate product of the gene encoding γ (*dnaX* [3, 11, 67]), is a DNA-dependent ATPase (30), the simplest interpretation is that δ stimulates the intrinsic ATPase activity of γ but is not itself an ATPase.

Whereas our analysis of the primary amino acid sequence of δ did reveal a possible ATP-binding site based on similarity to the Walker A-consensus ATP-binding site, several observations argue against the presence of a biologically active ATP-binding site in δ. First, the putative ATP-binding site of δ has 3 residues between the conserved Ala-1 and Gly-6, instead of the 4 residues found in the consensus motif. We were unable to find a single example of an ATP-binding protein with this deviation from the consensus. Second, the ATP-binding sites of previously characterized ATP-binding proteins almost always have a glycine residue at position 4 of the consensus. Thirty-five of the 37 ATP-binding proteins presented in references 16, 17, 45, and 69 have this glycine residue; the two exceptions, RecA and DnaB, have a serine. The ATP-binding site of δ contains neither a glycine nor serine residue at this position. Finally, the consensus ATP-binding site must form a loop between an α-helix and a β-sheet. Analysis of the secondary structure of δ by two different methods predicts that any ATP-binding site of δ is buried within a long α-helix. For these reasons, it is unlikely that δ contains an ATP-binding site. Studies to address this issue experimentally await purification of δ to homogeneity. Work is currently underway to purify δ from our overproducing strain and to examine the contribution of this subunit to the functional specialization of the two halves of the asymmetric, dimeric DNA polymerase III holoenzyme.

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